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(54) Title: SCREENING METHOD FOR PEPTIDES

(57) Abstract: The present invention relates to a method for screening nucleotide sequences encoding anti microbial peptides comprising the steps of: a) ligating a plasmid with the pool of nucleotide sequences operably linked to an inducible promoter, so as to express a peptide which is an enzyme or a mature peptide of less than 100 amino acid residues, optionally linked to a signal peptide, b) transforming host cells which are sensitive to the peptide with the ligated plasmids, c) screening the transformed host cells so as to select viable cells, d) cultivating the viable cells in the presence of inducer so as to induce expressions of said nucleotide sequence, e) selecting cells according to the effect of the inducer on cell growth, and recovering the nucleotide sequence encoding the peptide from the selected cells. DNA shuffling is emphasized as a method for producing the pool of nucleotides.

SCREENING METHOD FOR PEPTIDES

FIELD OF THE INVENTION

The present invention relates to a method for screening a pool of nucleotide sequences to select a nucleotide sequence encoding a peptide.

BACKGROUND OF THE INVENTION

Various bioactive peptides are known to kill or inhibit the growth of target cells, e.g. antimicrobial enzymes, anti-tumor peptides and antimicrobial peptides. An improved screening method for such peptides is desirable for the development of new bioactive peptides.

SUMMARY OF THE INVENTION

The object of the invention is to provide a method to identify novel or improve existing genes encoding bioactive peptides that can kill or inhibit the growth of target cells. The inventor has developed a suicide expression system (SES) for such peptides. The rationale of the SES is to generate libraries encoding peptides in cells, induce expression of the individual peptides, and select/identify peptide-encoding sequences according to their ability to kill or inhibit the growth of host cells as a result of synthesis of the peptide. Successive rounds of peptide induction, selection, plasmid amplification and mutagenesis can be used for the identification of peptides with improved bioactivity. However no protection or scaffold peptide is needed in this method to protect the active peptide from digestion within the cell. Such peptide may be needed for recovering and purifying the active peptide, but not

to identify the nucleotide sequence encoding the active peptide such as described in this invention.

Accordingly, the invention provides a method for screening a pool of nucleotide sequences to select a nucleotide sequence encoding a peptide, said method comprising:

(a) ligating a plasmid with the pool of nucleotide sequences operably linked to an inducible promoter, so as to express a peptide, which is an enzyme or a mature peptide of less than 100 amino acid residues, optionally linked to a signal peptide,

(b) transforming host cells which are sensitive to the peptide with the ligated plasmids,

(c) screening the transformed host cells so as to select viable cells,

(d) cultivating the viable cells in the presence of inducer so as to induce expression of said nucleotide sequence,

(e) selecting cells according to the effect of the inducer on cell growth, and

(f) recovering the nucleotide sequence encoding the peptide from the selected cells.

The rationale of the presented suicide expression system (SES) is to generate peptide libraries in microorganisms, induce expression of the individual peptides, and select/identify cells according to whether they are killed or severely growth inhibited as a result of sudden peptide synthesis.

For the identification of novel gene-encoded antimicrobial activities, libraries of genes harboring putative antimicrobial activities are cloned into the relevant plasmid, synthesis is induced, growth-inhibited or dead bacteria are identified and the corresponding gene sequenced and analyzed.

For the identification of variants of peptides with increased bioactivity, mutant libraries of an existing peptide is generated and introduced into the target organism. Successive rounds of peptide induction (using stepwise lower amounts of inducer), selection, plasmid amplification and shuffling/mutagenesis will allow the identification of peptides with improved bioactivity.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1.

Shows a flow diagram of the method step in a SES system using Fluorescence Assisted Cell Sorting (FACS) equipment for identification of modified Anti Microbial Peptides (AMP's), wherein A is a library of mutant Anti Microbial Peptides (AMP's) in bacterial host cells; B is FACS-mediated removal of dead bacteria; C is induction of transcription; D is FACS-mediated selection of non-viable bacteria and E is PCR amplification, shuffling of amplified genes, cloning and transformation.

The following symbols are used:

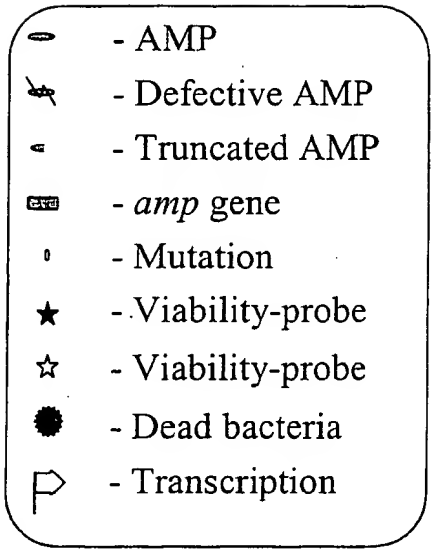


Figure 2

Shows a flow diagram of the screening strategy for conventional agar plates using solid media for identification of modified AMP's, wherein A is distribution of microbial clones on agar plates; B is making of a replica plate; C is induction of transcription and D includes characterization of colonies, such as properties, AMP sequence, identification of dead or inhibited cell colonies, PCR amplification, gene shuffling and re cloning

Figure 3.

Shows a flow diagram of the screening strategy for microtiter plates using liquid media for identification of modified AMP's, wherein A is distribution of microbial clones in micro titer wells; B is making of a replica plate; C is induction of transcription and D includes characterization of colonies, such as properties, AMP sequence, identification of dead or inhibited cell colonies, PCR amplification, gene shuffling and re cloning.

Figure 4.

Show the effect on E. coli transformed with DNA encoding AMP's, wherein the expression of the AMP's is inducible with an arabinose inducer. In the vertical direction levels of inducer are indicated. different AMP's are tested, wherein 1 is Andropin; 2 is Bac7; 3 is Bac5, 4 is StyelinD; 5 is StyelinC; 6 is PR39; 7 is ClavA; 8 is ClavAK; 9 is CAP18 and 10 is pBAD. the effects on the different E. coli colonies are visually detectable.

Figure 5.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Andropin (PHHA1000-Andropin), wherein the Andropin is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Andropin (PHH1000-Andropin), wherein the Andropin is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 6.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Bac7 (PHHA1100-Bac7), wherein the Bac7 is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Bac7 (PHH1100-Bac7), wherein

the Bac7 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 7.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Bac5 (PHHA1200-Bac5), wherein the Bac5 is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of Bac5 (PHH1200-Bac5), wherein the Bac5 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 8.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of StyelinD (PHHA1300-StyelinD), wherein the StyelinD is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of StyelinD (PHH1300-StyelinD), wherein the StyelinD7 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 9.

A: Growth-curves at different levels of arabinose inducer of *E.*

coli having induced expression of StyelinC (PHHA1400-StyelinC), wherein the StyelinC is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of StyelinC (PHH1400-StyelinC), wherein the StyelinC is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 10.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of PR39 (PHHA1500- PR39), wherein the PR39 is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of PR39 (PHH1500- PR39), wherein the PR39 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 11.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of ClavaninA (PHHA1600-ClavaninA), wherein the ClavaninA is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of ClavaninA (PHH1600-

ClavaninA), wherein the ClavaninA is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 12.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of ClavaninAK (PHHA1700-ClavaninAK), wherein the ClavaninAK is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of ClavaninAK (PHH1700-ClavaninAK), wherein the ClavaninAK is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 13.

A: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of CAP18 (PHHA1800-CAP18), wherein the CAP18 is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of CAP18 (PHH1800-CAP18), wherein the CAP18 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 14.

A: Growth-curves at different levels of arabinose inducer of *E.*

coli having induced expression of control peptide *Myc*/HIS6, wherein the *Myc*/HIS6 is kept in the cytoplasm. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

B: Growth-curves at different levels of arabinose inducer of *E. coli* having induced expression of control peptide *Myc*/HIS6, wherein the *Myc*/HIS6 is secreted to the periplasmic space. Growth is monitored by measuring OD at 450 nm of cell suspensions. Inducer levels are given in % w/w.

Figure 15.
Growth curves of *E. coli* having induced expression of StyelinC variants from randomly picked mutants. The randomly picked clones of mutant StyelinC is numbered from #1-#10. levels of inducer is given in %w/w.

DETAILED DESCRIPTION OF THE INVENTION

Peptide

The method of the invention may be used to screen peptides according to their bioactivity, i.e. their ability to kill or inhibit the growth of target cells. Thus, the peptide may be a peptide compound interacting/binding/sequestering essential cellular targets. The peptide of interest may be an antimicrobial enzyme or a short peptide (less than 100 amino acid residues), e.g., an anti-microbial peptide (AMP) or an anti-tumor peptide.

The antimicrobial enzyme may be, e.g., a muramidase, a lysozyme , a protease, a lipase, a phospholipase, a chitinase, a glucanase, a cellulase, a peroxidase, or a laccase. Alterna-

tively, a consortium of enzymes synthesizing conventional antibiotics, e.g. polyketides or penicillins, can be employed.

The antimicrobial peptide (AMP) may be, e.g., a membrane-active antimicrobial peptide, or an antimicrobial peptide affecting/interacting with intracellular targets, e.g. binding to cell DNA. The AMP is generally a relatively short peptide, consisting of less than 100 amino acid residues, typically 20-80 residues. The antimicrobial peptide has bactericidal and/or fungicidal effect, and it may also have antiviral or antitumour effects. It generally has low cytotoxicity against normal mammalian cells.

The antimicrobial peptide is generally highly cationic and hydrophobic. It typically contains several arginine and lysine residues, and it may not contain a single glutamate or aspartate. It usually contains a large proportion of hydrophobic residues. The peptide generally has an amphiphilic structure, with one surface being highly positive and the other hydrophobic.

The bioactive peptide and the encoding nucleotide sequence may be derived from plants, invertebrates, insects, amphibians and mammals, or from microorganisms such as bacteria and fungi.

The antimicrobial peptide may act on cell membranes of target microorganisms, e.g. through nonspecific binding to the membrane, usually in a membrane-parallel orientation, interacting only with one face of the bilayer.

The antimicrobial peptide typically has a structure belonging to one of five major classes: α helical, cystine-rich (defensin-like), β-sheet, peptides with an unusual composition

of regular amino acids, and peptides containing uncommon modified amino acids.

Examples of alpha-helical peptides are Magainin 1 and 2; Cecropin A, B and P1; CAP18; Andropin; Clavanin A or AK; Styelin D and C; and Buforin II. Examples of cystine-rich peptides are α-Defensin HNP-1 (human neutrophil peptide) HNP-2 and HNP-3; β-Defensin-12, Drosomycin, γ1-purothionin, and Insect defensin A. Examples of β-sheet peptides are Lactoferricin B, Tachyplesin I, and Protegrin PG1-5. Examples of peptides with an unusual composition are Indolicidin; PR-39; Bactenicin Bac5 and Bac7; and Histatin 5. Examples of peptides with unusual amino acids are Nisin, Gramicidin A, and Alamethicin.

Another example is the antifungal peptide (AFP) from *Aspergillus giganteus*.

In a preferred embodiment the expressed peptide is free of any protecting scaffold proteins.

Pool of nucleotide sequences

The commercial utility of antimicrobial peptides as antibiotics or antimicrobial agents depends on their potency, species specificity and ability to perform under the appropriate conditions. More often than not, these conditions are quite different from those under which the peptide originally evolved. Most antimicrobial peptides have, for example, not been evolved to simultaneously target a broad range of different microbes, to work in a physiological salt range, to evade the human immune system or resist the clearing capacity of the mammalian circulatory system.

For a given antimicrobial peptide, this dilemma can in principle be solved by either knowledge-based rational modifi-

cations of the peptide or by directing further the evolution of the peptide, creating random variants of the parental sequence, and subsequently selecting mutants in which the desired combination of properties are found. Directed evolution, an iterative process by which large areas of sequence space are explored to create mutant proteins and peptides that possess particular desired characteristics, combined with powerful High Throughput assays allows large libraries of native or modified gene-encoded antimicrobial peptides to be generated and evaluated for the identification of lead candidates with the desired characteristics. These approaches are now being adopted widely by academics and the industry alike to create novel protein-based activities at an unprecedented rate.

A nuclectide sequence, which encodes the bioactive peptide, may be obtained from chromosomal DNA from on of the above-mentioned source organisms and/or it may be chemically synthesized. The nucleotide sequence may also be a cDNA derived from such source organisms.

The screening method of the invention may be used to develop peptides with an improved bioactivity. Thus, starting with a known gene encoding a bioactive peptide, a DNA pool may be obtained, e.g., by random mutagenesis to produce a mutant library, by gene shuffling, or by synthesizing degenerate genes.

The sequences to be shuffled may be related sequences from different organisms (so-called "family shuffling"), or they may include a parent sequence and a variant thereof.

In a preferred embodiment of the invention random mutagenesis is achieved by shuffling of homologous DNA sequences in vitro such as described by Stemmer (Stemmer, 1994.

Proc. Natl. Acad. Sci. USA, 91:10747-10751; Stemmer, 1994. Nature 370:389-391) and Crameri, A., et al., 1997. Nature Biotechnology 15:436-438 all incorporated by reference. The method relates to shuffling homologous DNA sequences by using in vitro PCR techniques. Positive recombinant genes containing shuffled DNA sequences are selected from a DNA library based on the improved function of the expressed proteins.

The above method is also described in WO 95/22625, hereby incorporated by reference, in relation to a method for shuffling homologous DNA sequences. An important step in the method is to cleave the homologous template double-stranded polynucleotide into random fragments of a desired size followed by homologously reassembling the fragments into full-length genes.

In another preferred embodiment of the invention random mutagenesis is achieved by the method described in WO 98/41653, incorporated by reference, which discloses a method of DNA shuffling in which a library of recombined homologous polynucleotides is constructed from a number of different input DNA templates and primers by induced template shifts during in vitro DNA synthesis. In this context especially the special version of this in vitro recombination through induced template shifts during DNA synthesis, also described in WO 98/41653, is preferred. Here, small (>5 nucleotides) random DNA primers are employed to randomly initiate DNA synthesis on the mutant DNA templates that are to be combined.

Due to the small size of the genes encoding antimicrobial peptides, special attention has to be taken into consideration when using each of the above methods for generation and combination of sequence diversity. Since most shuffling methods rely

on a substantial number of identical bp (20-100 bp) flanking the mutations that has to be recombined, the mutations in small genes are technically difficult to combine by the above described methods.

Accordingly, other formats of directed evolution have to be employed on small genes. In a preferred embodiment involving the combination of variants of a given peptide of less than approximately 50 amino acids, one degenerate DNA primer harboring all the desired mutations would be synthesized. In a given position in this degenerate primer, both the wt nucleotide as well as the mutant nucleotide should be present. The frequency of wt-to-mutant nucleotides can be adjusted as considered optimal; rules and considerations are known in the art. By including all desired mutations in one primer, the desired sequence-space could be completely sampled. This method allows for the sampling and combination of all desired mutations irrespectively of how close they would be in the primary gene sequence.

If peptides of more than approximately 50 amino acids are employed, two or more separate and degenerate primers would have to be used. This is due to the constraints generally experienced when synthesizing DNA primers; only DNA primers of less than approximately 180-200 nucleotides can routinely be synthesized.

In another embodiment where peptides longer than approximately 50 amino acids are employed, the sequence diversity (the individual mutants) to be combined can individually be harbored in small oligonucleotides of 20-30 base pairs of length. In this approach, a specific DNA oligo is employed for each mutation that should be included in the library. The mutations should preferentially be located in the middle of the small

oligo to optimize annealing. Spiking in several or numerous of these small oligoes in a PCR reaction using the wt peptide gene as backbone for the amplification, would allow for the combination of the desired mutations. By varying the amount of the individual oligoes to be combined, desired ratios of individual variants to wt's can be created. As approximately 10 base pairs is required on each side of the sequence mismatch, this method cannot effiently combine mutations that are immediately adjacent.

The Suicide Expression System is not limited to the identification of improved variants of existing and already characterized peptides. New genes encoding peptides that affects the growth of a given host cell can also be identified. Libraries of cDNA's or randomly generated whole-genome DNA fragments can be employed as starting material and cloned into the Suicide Expression System.

Host cell

The host cell must be sensitive to the peptide, enzyme or secondary metabolite of interest.

Thus, in the case of screening for an antimicrobial peptide (AMP) or an antimicrobial enzyme, the host cell can be a bacterium such as *E. coli* or *Bacillus*, e.g. *B. subtilis*, or the host cell can be a fungal cell, e.g. a filamentous fungus such as *Aspergillus* or a yeast, such as *Saccharomyces* or *Candida*. It may be preferred to use a host cell related to the target microorganisms against which the antimicrobial peptide is intended to be used.

In the case of screening for an anti-tumor peptide, the host cell is preferably a mammalian cell, particularly a tumor cell.

The host cell should be capable of transporting the inducer across the membrane preferably without metabolizing or degrading it. This is advantageous for expression studies as the level of inducer will be constant inside the cell and not decrease over time. This can be achieved by selecting a "gratuitous" inducer, or it can be achieved by deleting one or more genes necessary for metabolism of the inducer.

The host cell must be selected so as to be able to express the antimicrobial peptide. Thus, a fungal cell is preferred for peptides with disulfide bridges such as the cystine-rich peptides mentioned above.

Plasmid

The plasmid should be replicable in the host organism, and should be able to express the bioactive peptide (and optionally signal peptide) under the control of the inducible promoter. It will usually contain a selectable marker such as an antibiotic marker. A number of such plasmids are known in the art.

Ligation

The plasmid is ligated with the pool of nucleotide sequences so that these sequences may be operably linked to an inducible promoter in the plasmid, enabling inducible expression of the peptide of interest, optionally linked to a signal peptide. The short peptide or enzyme of interest may be expressed without any extension (other than the optional signal

peptide), or it may be expressed with a short extension of, e.g., 1-5 amino acids. Expression in the form of a fusion protein is neither preferred nor necessary.

Inducible promoters and inducers

The plasmid to be used according to the invention must comprise an inducible promoter regulating the expression of the inserted nucleotide sequence encoding the peptide. It is advantageous for the applicability of the SES, that it allows a complete shutdown of the synthesis of the encoded bioactive peptide. In addition, the induction of the encoded bioactive peptides should be significant, since peptides are inherently unstable and easily degraded in the cytoplasm of microorganisms. The inducible promoter employed in the current examples is both positively and negatively regulated by two proteins. In the presence of inducer, expression from the promoter is turned on, while in the absence of inducer, very low levels of expression occur from the promoter. Uninduced levels are repressed even further by growth in the presence of a secondary metabolite. By varying the activity of the two regulators, protein expression levels can be manipulated to optimize expression of potentially toxic or essential genes.

The promotor may be the Lac promotor as descibed in Taguchi S., Nakagawa K., Maeno M. and Momose H.; "*In Vivo Monitoring System for Structure-Function Relationship Analysis of the antibacterial peptide Apidaecin*"; Applied and Environmental Microbiology, 1994, pp. 3566-3572, which may be regulated by presence of the inducer lactose or by the synthetic non-digestible lactose derivative IPTG. Other inducible promoters are known in the art such as *trp* promoters induced by trypto-

phan or *gal* promoters induced by galactose for *E. coli*, *gal1* promoter for *S. cerevisiae*, *AOX1* promoter for *Pichia pastoris*, *pMT* (metallothionein) promoter for *Drosophila*, MMTV LTR , pVgRXR or pIND promoters for mammalian expression. Using an inducer that is not metabolized or digested in the cell offers the advantage that the inducer concentration may be kept constant throughout the screening process. However a drawback of the Lac promoter may be that it cannot be entirely switched off by the absence of the inducer. The promoter may also be the pBAD promoter as used in the examples, *vide infra*. This promotor is, *inter alia*, induced by the digestible inducer arabinose. However to achieve the above mentioned advantage of having a constant level of inducer, the host cells ability to digest arabinose can be eliminated by deleting suitable genes from the host cell genome (a description of the genotype may be found in the examples). An important consideration selecting a suitable promotor is however that the corresponding inducer should be able to permeate the cell membrane(s) to gain access to the promoter.

The pBAD promoter is both positively and negatively regulated by two proteins, AraC and cAMP-CRP. In the presence of arabinose, expression from the promoter is turned on, while in the absence of arabinose, only very low levels of expression occur from the promoter.

Uninduced levels are repressed even further by growth in the presence of glucose. Glucose acts by lowering cAMP levels, which in turn decreases the binding of cAMP-CRP to the promoter region of pBAD. As cAMP levels are lowered, transcriptional activation is decreased. This is ideal when the peptide of interest is extremely growth inhibitive or toxic to the host. In

conclusion, by varying the activity of the two regulators, protein expression levels can be manipulated to optimize expression of potentially toxic or essential genes.

Signal peptide

A DNA sequence encoding a signal peptide may optionally be inserted into the plasmid downstream of the inducible promoter and upstream of the sequence encoding the peptide, so that the antimicrobial peptide will be expressed with the signal peptide attached. A suitable signal peptide for a given host cell may be selected according to principles known in the art.

Generally, the peptide of interest initially attacks or penetrates the target organism from the outside, so the success of the SES will in most cases require that the peptide in question is exported out of the cell. In general, the bioactive peptide will be secreted through the cell membrane, e.g. to the periplasmic space in gram-negative prokaryotes, and from there allowed to interact with its cellular target, e.g. the cellular membranes, components in the membranes or the periplasmic space, or allowed to further diffuse through the outer membrane.

A signal peptide can be omitted if the peptide of interest can exert its action when expressed and retained within the cell, e.g. peptides that bind to the cellular DNA or peptides that do not depend on a trans-membrane potential or peptides with intracellular targets. An example is the family of proline-arginine-rich peptides Bac5, Bac7 and PR39, which in the literature have been suggested to interact and sequester nucleic acids.

Screening process

As mentioned above the invention relates to a method for screening a pool of nucleotide sequences to select a nucleotide sequence encoding an antimicrobial peptide which acts on cell membranes, cell walls or DNA of target microorganisms, said method comprising the steps of:

(a) ligating a plasmid with the pool of nucleotide sequences operably linked to an inducible promoter, so as to express a peptide which is an enzyme or a mature peptide of less than 100 amino acid residues, optionally linked to a signal peptide,

(b) transforming host cells which are sensitive to the peptide with the ligated plasmids,

(c) screening the transformed host cells so as to select viable cells,

(d) cultivating the viable cells in the presence of inducer so as to induce expression of said nucleotide sequence,

(e) selecting cells according to the effect of the inducer on cell growth, and

(f) recovering the nucleotide sequence encoding the peptide from the selected cells.

Prior to step a) of the screening process certain preparatory steps may be necessary. A host, for which a killing and/or growth inhibiting peptide is desired to be found and a suitable plasmid compatible with that host should be chosen. A library of nucleotide sequences, such as a pool of nucleotide sequences derived by mutating a known sequence encoding a known

antimicrobial peptide, should be prepared, e.g. by conventional methods, such as described *vide supra*.

In step **a)** the library is ligated to the suitable plasmid and transformed (**step b**) into the host cell culture by conventional methods.

Step **c)** is a first screening or selection step, in which viable cells are separated from cells which died and/or became growth inhibited during the cause of the transformation process. This step is an important one because the ultimate goal in the screening process is to identify cells that dies and/or is growth inhibited by the induced expression of the inserted nucleotide sequence producing an antimicrobial peptide. Cells which death and/or inhibition occurred before the screening process and thus is not caused by the antimicrobial peptide would generate a false positive response in the screening if they were not separated from the viable cells. In step **d)** an inducer is introduced to the viable cells, which are cultivated so as to induce expression of the nucleotide sequence from the library comprised in the inserted plasmid. As the peptide is produced by transcription the host cell will die and/or be growth inhibited if said peptide has antimicrobial effect against the host. In a preferred embodiment host cells which are dead and/or growth inhibited are selected. By selection of dead and/or growth inhibited host cells, cell comprising nucleotide sequences encoding peptides having antimicrobial activity may be isolated. More than one level of inducer concentration may be employed in parallel so that a graduated response may be achieved and nucleotide sequences encoding peptides having different antimicrobial effects or potency may be identified. In step **e)** host cells which die and/or becomes

growth inhibited are selected and separated from host cells which are unaffected by the peptide expressed from the plasmid nucleotide sequence which was inserted under the transformation of the host cells. One may select only cells which are greatly affected by the induced expression of a peptide, e.g. which are affected by small concentrations of inducer, or one may select all affected cells, depending on the intended scope of the screening and/or the existing knowledge of the pool or library of nucleotide sequences.

The criterion on which a cell is selected may be chosen individually, e.g. a maximum inducer concentration may be set so that only cells which are affected by the presence of inducer below this inducer concentration are selected, and/or decreasing levels of transcriptional induction using incremental lower concentrations of inducer on replicas of the transformed host cells will allow the isolation of peptides with increased bioactivity (figure 4).

The inserted nucleotide sequences from the selected host cells identified as encoding bioactive peptides may be recovered by conventional methods. The nucleotide sequences may be amplified by conventional methods, e.g. PCR amplification. From here an identified and amplified nucleotide sequence may be inserted into a production host and the corresponding peptide identified may be mass produced according known methods where a peptide may be expressed through fusion to a bigger polypeptide which then may be exported by the host cell. Said polypeptide may have the function of protecting the peptide of interest from digestion within the cell and thereby inactivation by the host cell enzymes and/or the polypeptide may have the function of lowering the effect of the peptide on the host cell so that

the host may proliferate and continue expression of the peptide without being significantly affected by the expressed peptide, an effect which would occur if the peptide had not been incorporated into the polypeptide. The identified and amplified nucleotide sequence encoding the peptide may also be mutated as described, *vide supra*, e.g., by random mutagenesis, by gene shuffling, or by synthesizing degenerate genes. These new mutated nucleotide sequences may then be screened again according to steps a) to f) to identify nucleotide sequences encoding new peptides with an improved effect e.g. by lowering the concentration of inducer in subsequent screenings.

The screening or separation processes may in a specific embodiment be conducted by application of conventional plate assays, so that the transformed host cells are streaked out on plates comprising a nutrient medium and optionally an antibiotic. If the transformation plasmid comprises a gene for resistance to such an antibiotic untransformed host cells will die on such a medium while transformed host cells will survive. The plates are then incubated for a predetermined period of time to enable colony formation of transformed host cells and from these plates cell samples are transferred to other plates further comprising an inducer inducing expression and production of the peptide comprised in the inserted plasmid. If a transformed host cell keeps growing and forms normal colonies in this environment it may be deduced that the expressed and produced peptide do not kill and/or inhibit the host cell. If on the other hand the host cell does not form any colonies or reduced colonies as compared to normal growth, it is evident that the induced peptide has an antimicrobial effect on the host cell. A depiction of the screening strategy for conventional

agar plates is given in figure 2.Plate assays, however, involves time consuming and tedious procedures and in a more preferred embodiment the screening or separation processes are performed in microtiter assays as described in the art. In this type of assay the liquid host cell culture is placed with a single or only a few cells comprising different inserted nucleotide sequences in each well of microtiter plates or otherwise securing that each well comprise only a single or a few nucleotide sequences to be investigated (e.g. a large number of host cells comprising the same inserted nucleotide sequence). The host cells in each well may be cultivated by addition of a nutrient medium and a copy or replicas of the microtiter plates may be prepared by transferring subsamples to additional testing plates. A medium containing an inducer may then be added to each well and the proliferation of the host cell culture in each well upon cultivation may be monitored, e.g. by measuring the optical diffraction through the cell suspension in each well. If a host cell grows unaffected of the expressed peptide the number of cells in this well will increase normally and the optical diffraction of the cell suspension measured through the well will increase. If, however, the host cell growth is affected of the expressed peptide the number of cells in this well will be lowered as compared to normal growth and the change in optical diffraction of the cell suspension will also be lowered. A depiction of the screening strategy for microtiter plates is given in figure 3.As a third and most preferred embodiment the screening or separation processes may be performed by employing Fluorescence Assisted Cell Sorting (FACS) equipment such as described in Gant V.A., Warnes G., Phillips I. and Savidge G.F.; "*The application of flow cytometry to the*

study of bacterial responses to antibiotics"; J. Med. Microbiol.; 1993; 39; pp. 147-154. This type of equipment is extensively described in the art, e.g. by the manufacturers of such equipment. Using this approach a viability probe, e.g. a fluorescent or colorimetric probe) is incorporated in the host cells, the probe being an indicator of the proliferation of each cell. Suitably the inducer and viability probe is added to an exponentially growing liquid culture of host cells, and the dead or growth-inhibited microorganism is identified and collected.

Having a such probe incorporated the viability of a cell may be monitored by measuring the fluorescence of the probe in the cell by exposing the cell with excitation light of a wavelength suitable for the probe, e.g. if fluorescence of the probe can be measured the cell is alive or vice versa. With a FACS machine cells which exhibits the desired characteristics may be selected at a tremendous speed and accuracy also aided by the fact that fluorescence measurements are highly sensitive. In the method of the invention FACS equipment may be employed in the screening or selection step c) where viable and transformed host cells are selected and/or e) where dead and/or inhibited cells are selected after inducing expression of the peptide or the FACS equipment may be combined with plate and/or microtiter plate techniques as described *supra*. Many suitable fluorescent probes are commercially available for this purpose, e.g. from Molecular Probes, Inc, Eugene, OR, USA. Using such probes it may be monitored whether e.g. the membrane-structure is compromised or deteriorated, whether the cross-membrane potential is reduced or eliminated, or whether specific probes are allowed to interact with intracellular targets (*e.g.* DNA).

Examples of such probes include but is not limited to SYTO(X)® nucleic acid stains from Molecular Probes, Inc. which are probes which are designed to either penetrate dead and/or damaged cells and make nucleic acids within such cells fluorescent (e.g. SYTOX® Green nucleic acid stain) or it may be designed to penetrate and make fluorescent living cells (e.g. SYTO® live-cell nucleic acid stains). The procedures for using such probes are available from the manufacturer. Also fluorescent redox probes (sensitive towards the cross membrane potential may be employed as described in Rodriguez, G.G., Phipps D., Ishiguro K. and Ridgway H.F.; *"Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria"*, Applied and Environmental Microbiology, 1992, pp. 1801-1808, wherein a 5-cuano-2,3-ditolyltetrazolium chloride probe is employed or in Jepras R.I., Paul F.E., Pearson S.C. and Wilkinson M.J.; " *Rapid Assessment of Antibiotic Effects on Escherichia coli by bis-(1,3-Dibutylbarbituric Acid) trimethine Oxonol and Flow cytometry*; Antimicrobial Agents and Chemotherapy; 1997; pp. 2001-2005, wherein a $DiBAC_4(3)$ probe available from Molecular Probes is employed.

FACS equipment or conventional luminiscence equipment may also be adapted to the use of bioluminiscence, a technique described in Virta M, Åkerman K.E.O., Saviranta P., Oker-Blom C. and Karp M.T.; " *Real time measurement of cell permeabilization with low-molecular-weight membranolytic agents"*, Journal of Antimicrobial Chemotherapy; 1995; 36; pp. 303-315. In addition, colorimetric indicators may be applied such as described in Roslev P. and King G.M.; " *Application of a Tetrazolium Salt with Water-Soluble Formazan as an Indicator of viability in Respiring Bacteria"*; Applied and Environmental Microbiology,

1993, pp. 2891-2896; although such indicators may more suitable be employed non-fluorescence cell sorting equipment designed for colorimetric measurements. A depiction of the screening strategy for FACS is given in figure 1.

Applying the method of the invention for screening for bioactive peptides in cells, in particular eukaryotic cells, e.g. mammalian cells, an alternative preferred screening or selection strategy may be employed. Upon the induced expression of the inserted nucleotide sequence the resulting peptide may, if it is bioactive against the host cell, disrupt and/or deteriorate the cell membrane and cells killed and/or affected cells may be separated from unaffected cells by centrifugating or filtering the cell suspension. If centrifugating the unaffected living cells will precipitate while nucleic acid material from affected cells may be isolated in the supernatant. This separation may also be achieved by filtering off the unaffected living cells. From this separation step amplification and mutation can be carried out as described *vide supra*.

Once the best peptides has been identified other tests may be performed, wherein the peptide sensitivity towards salt concentration, ionic strength, pH and/or and especially sensitivity of normal mammalian cells toward the peptides or other relevant parameters and conditions are tested depending of the intended application of the peptides.

Use of antimicrobial peptide

The antimicrobial peptides found from the method of the invention may be employed in many areas of application. One suitable area is preservation of e.g. of food/feed, paint formulations, detergent formulations, cosmetics or other personal care prod-

ucts as an alternative to chemical preservatives. The peptides may also be used to preserve medical devices such as prosthetic implants, intravenous tubing e.g. by coating such materials with a coating comprising said peptides. The peptides may also be actively applied to disinfect and/or kill and/or inhibit microbial cells on an object e.g. in the cleaning industry, e.g. as an disinfectant for treatment of biofilm. One preferred application is the preparation of peptides for treating microbial infections and/or tumors in the human and/or animal body or on the skin or mucous membranes. It is contemplated that the use of the screening method of the invention is a versatile tool for finding extremely bioactive peptides which is able of killing and/or inhibiting microbial and/or tumor cell, but which have little or no negative effect on normal mammalian and/or eukaryotic cells. The peptides may be formulated for oral administration or for intravenous or subcutaneous injection or as an ointment.

EXAMPLES

Example 1: Growth inhibiiton of *E. coli* upon expression of various antimicrobial peptides

Genes of model AMP's, i.e. CAP18, PR39, Andropin, Bac5, Bac7 ClavaninA, Clavanin AK (a Clavanin A variant), Styelin D and Styelin C, were synthesized using DNA oligoes in a standard PCR reaction and cloned in the presented SES in order to evaluate its potential for identifying AMP's with improved bioactivity.

Plasmid

Two series of experiments were made: Series pHH (using plasmid pBAD/gIIIA) allowed for the export of the AMP's to the periplasmic space of *E. coli*, from where the peptides were allowed to interact with the cellular membranes. In series pHHA (using plasmid pHHA) the peptides lacked a signal sequence and correspondingly were retained in the cytoplasm.

One of the parental plasmids employed, pBAD/gIIIA, is commercially available from Invitrogen. It is a pUC-derived expression vector designed for tightly regulated, recombinant protein expression in *E. coli*. This plasmid allows the cloning of peptides and proteins toxic to *E. coli*, as no expression of the recombinant peptides occurs in the absence of inducer in the growth medium. However, transcription and hence peptide synthesis, can be extensively induced.

Secretion

As all AMP's initially attacks or penetrates the target organism from the outside, the success of the SES will in most cases require that the AMP in question is exported out of the cell. In the current system, the AMP was secreted to the periplasmic space, and from there allowed to interact with its cellular target, *e.g.* the cellular membranes, components in the membranes or the periplasmic space, or allowed to further diffuse through the outer membrane.

In series pHH, the gene III signal sequence in pBAD/gIIIA was located in front of the inducible promoter in order to mediate secretion of the peptide/protein in question. Gene III encodes pIII, one of the minor capsid proteins from the filamentous phage fd. pIII is synthesized with an 18 amino acid, N-

terminal signal sequence, and requires the bacterial Sec system for insertion into the membrane. The signal sequence was removed after crossing the inner membrane, thus leaving the native peptide. A *Nco*I restriction site immediately succeeds the signal sequence cleavage site.

In the other parental plasmid series, pHHA, the peptides were synthesized without a signal sequence, and correspondingly, were retained in the cytoplasm. The pHHA plasmid differs only from pBAD/gIIIA, in that the gene III has been deleted. This deletion was produced by the introduction of an additional *Nco*I site overlapping the translation initiation site. Hence, the signal sequence was removed by restriction with *Nco*I, and the plasmid religated to produce pHHA.

In both plasmid systems (pBAD/gIIIA and pHHA), the AMP genes are inserted as a *Nco*I-*Xba*I fragment.

Regulation of the inducible promoter

The inducible promoter employed, pBAD, is both positively and negatively regulated by two proteins, AraC and cAMP-CRP. In the presence of arabinose, expression from the promoter is turned on, while in the absence of arabinose, very low levels of expression occur from the promoter. Uninduced levels are repressed even further by growth in the presence of glucose. Glucose acts by lowering cAMP levels, which in turn decreases the binding of cAMP-CRP to the promoter region of pBAD. As cAMP levels are lowered, transcriptional activation is decreased. This is ideal when the peptide of interest is extremely growth inhibitive or toxic to the host. In conclusion, by varying the activity of the two regulators, protein expression levels can

be manipulated to optimize expression of potentially toxic or essential genes.

C-terminal myc epitope and 6xHis tag

The various AMP genes mentioned above, were cloned in front of an in-frame *myc* epitope and 6xHis tag. A TAG stop codon separated the AMP gene and the sequence encoding the two epitopes. This means that in normal *E. coli* cells, only the AMP is synthesized upon transcriptional induction. Translational termination at the TAG stop codon can, if the fusion protein is non-toxic to *E. coli*, be suppressed in various strains, allowing for synthesis of an AMP/*myc*/6xHis fusion protein. This fusion protein is easily purified using affinity nickel (Ni^{2+}) resins, and is easily detected using anti-*Myc* or anti-6xHis antibody. Following purification, CnBr cleavage will separate the two tags from the AMP by cleavage at a conveniently located methionine. This system allows for an easy, convenient purification of selected peptides allowing for confirmation, using traditional assays, of the antimicrobial activity.

Host organism

The strain employed was *E. coli* TOP10 (commercially available from Invitrogen). It is *ara*BADC$^-$ and *ara*EFGH$^+$.

AMP genes

The active fragments of the following model AMP's, *i.e.* CAP18, PR39, Andropin, Bac5, Bac7 ClavaninA, ClavaninAK (a ClavaninA variant), StyelinD and StyelinC, were synthesized in a standard PCR reaction using the DNA oligoes shown below. The PCR amplified AMP genes were restricted with *Nco*I and *Xba*I, and ligated into the corresponding cloning sites in pBAD/gIIIA and

pHHA. These ligation mixtures were transformed into chemically competent *E. coli* TOP10, and individual clones analyzed. The AMP genes were finally verified by DNA sequencing.

Primers used for synthesis of the AMP genes.

Andropin

ccggccatggtatttattgatattcttgacaaagtggaaaacgcaatacacaatgctgctcaa
gtgggaattggctttgctaagccctttgaaaaattgatcaatccgaagtagatggctctagac
ggc

PR39

ccggccatggcgaggagacgtccccgacccccatatttgccaaggccaaggccacctccgttt
ttcccaccaaggctcccaccaaggatcccaccagggttcccaccaaggttcccaccacggttc
cccggaaaacggtagatggctctagacggc

Bac5

ccggccatggcgagatttcgtccaccaatccgtcgtccaccaatccgtccgccgttctatcca
ccgttccgcccgccgatccgcccaccgatcttcccaccgatccggccaccgttccgtccaccc
ttaggaccgtttcctggtagacggtagatggctctagacggc

Bac7-Forw

ccggccatggcgaggagaattcgtccccggccaccacgtttgccaaggccaaggccaaggcca
ttgccattcccacggcctgggccaaggccaattccaaggccactgccattcccacggcctggg
ccaaggccaattccaaggccactg

Bac7-rev

gccgtctagagccatctacaatggccttggaattggccttggcccaggccgtgggaatggcag
tggccttggaattggccttggcccaggccgtgggaatggcagtggccttggaattgg

CAP18

ccggccatggggctgcgcaagcgcttacgaaaatttagaaacaagattaaagaaaagcttaaa
aaaattggtcagaaaatccagggtttcgtgccgaaacttgcacccaggacagattactagatg
gctctagacggc

Clavanin A

ccggccatggtattccaattccttggcaaaattattcatcatgttggcaattttgtacatggt
tttagccacgtgttttagatggctctagacggc

Clavanin AK

ccggccatggtattccaattccttggcaaaattattaagaaggttggcaattttgtaaagggt
tttagcaaggtgttttagatggctctagacggc

Styelin C

ccggccatgggctggtttggaaaagctttcagatcagtaagcaacttttacaaaaaacataaa
acatacatccatgcaggactttcagctgctacattgcttggttagatggctctagacggc

Styelin D

ccggccatgggttggttgagaaaagctgccaaatctgtaggaaaattttactacaaacacaaa
tattacatcaaagcagcctggcaaattggaaagcatgccttaggttagatggctctagacggc

The corresponding amino acid sequences of the AMP genes are shown below. The amino acids in lower case are not present in the native AMP's but were introduced as a result of the cloning strategy where *NcoI* is employed as the proximal cloning site (CCATGG; ATG encodes methionine). The natural codon usage of the genes have been retained.

CAP18 mGLRKRLRKFRNKIKEKLKKIGQKIQGFVPKLAPRTDY

PR39 maRRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPGKR

Andropin mVFIDILDKVENAIHNAAQVGIGFAKPFEKLINPK

Bac5 maRFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFPGRR

Bac7 maRRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPLPF-
PRPGPRPIPRPL

ClavaninA mVFQFLGKIIHHVGNFVHGFSHVF

ClavaninAK mVFQFLGKIIKKVGNFVKGFSKVF

StyelinC mGWFGKAFRSVSNFYKKHKTYIHAGLSAATLLG

StyelinD mGWLRKAAKSVGKFYYKHKYYIKAAWQIGKHALG

Growth Inhibition of *E. coli* upon Expression of Various AMP's

The following experiment was conducted in order to evaluate whether E. coli, upon induction of endogenous AMP expression, was growth-inhibited in liquid media.

On day one, cells harboring the various AMP-encoding plasmids, were inoculated in LB + 100 γ ampicillin and grown under non-induced conditions at 37°C with vigorous shaking. These overnight cultures were on day two diluted 100-fold into fresh, pre-warmed LB broth + 100 γ ampicillin, in the presence of varying amounts of arabinose (0%; 0.001%, 0.01% and 0.1%). 100 µl of these freshly diluted cultures were transferred to microtiter plates, and incubated at 37°C with vigorous shaking. Growth of the cultures were measured at regular intervals at OD_{450} using an ELISA reader. The corresponding growth-curves are shown in figures 5-14.

In the pHH-series (using the parental plasmid pBAD/gIIIA), where the peptides are secreted to the periplasmic space, expression of all AMP's except Andropin, significantly inhibited the growth of the bacteria. Similar results were obtained when other strains of *E. coli* with similar genotype

(*ara*BADC$^-$ and *ara*EFGH$^+$) were employed (Data not shown). Androdopin has been reported to require substantial concentrations of salt in order to fold and exert antimicrobial activity; an observation that can explain the less potent inhibition seen. No growth inhibition is evident in strains carrying the control plasmid pBAD/gIIIA expressing the 38 amino acid *Myc*/HIS6 control peptide fused to the pIII signal sequence. Hence, it can be concluded that expression of peptides fused to the pIII signal sequence *per se* does not significantly inhibit the growth of *E. coli*. The results are shown in Figs. 5b - 14b.

In the pHHA-series, where the peptides were retained in the cytoplasm, a different pattern of growth inhibition was observed. Only a subset of the AMP exerted growth-inhibition in this context. This difference most likely reflects differences in mode of action of the AMP's. Peptides that do not depend on a trans-membrane potential or peptides with intracellular targets (*e.g.* the family of proline-arginine-rich peptides Bac5, Bac7 and PR39 have in the literature been suggested to interact and sequester nucleic acids) would be expected to affect the viability when expressed and retained within the cell. The results are shown in Figs. 5a - 14a.

Growth Inhibition of *E. coli* on Solid Media upon Expression of Various AMP's

The following experiment was conducted in order to evaluate whether *E. coli*, upon induction of endogenous AMP expression, was growth-inhibited on solid media.

On day one, cells harboring AMP-encoding plasmids, were inoculated in LB + 100 γ ampicillin and grown under non-induced conditions at 37°C with vigorous shaking. These overnight cul-

tures were then on day two diluted 100-fold into fresh, pre-warmed LB broth + 100 γ ampicillin, and 3 µl spotted onto LB agar plates containing 100 γ ampicillin and varying amounts of arabinose (0%; 0.001%, 0.01% and 0.1%). These agar plates were then incubated over night at 37°C. The growth bacterial clones were recorded the following day. Inhibition of growth was observed to correlate with the amount of arabinose present. The pattern of inhibition reflected the results observed when grown in liquid broth.

Mutant libraries

In order to examine whether the suicide expression system is able to distinguish among peptide variants displaying different antimicrobial activity, mutant libraries of the 9 AMP genes were generated using PCR and 0.5 mM $MnCl_2$. For illustrative purposes, randomly picked clones of mutant StyelinC clones have been selected and analyzed for inducer-dependent growth-inhibition. Mutant AMP's have been identified with what appears to be altered bioactivity (Fig. 15). A subset of the mutants were sequenced, and all clones displaying altered bioactivity were found to be different from the *wt* (wild-type) in question.

CLAIMS

1. A method for screening a pool of nucleotide sequences to select a nucleotide sequence encoding a peptide, said method comprising:

a) ligating a plasmid with the pool of nucleotide sequences operably linked to an inducible promoter, so as to express a peptide which is an enzyme or a mature peptide of less than 100 amino acid residues, optionally linked to a signal peptide,

b) transforming host cells which are sensitive to the peptide with the ligated plasmids,

c) screening the transformed host cells so as to select viable cells,

d) cultivating the viable cells in the presence of inducer so as to induce expression of said nucleotide sequence,

e) selecting cells according to the effect of the inducer on cell growth, and

f) recovering the nucleotide sequence encoding the peptide from the selected cells.

2. The method of claim 1 wherein the pool of nucleotide sequences is produced by random mutagenesis, by gene shuffling or by synthesizing degenerate genes.

3. The method of claim 1 or 2 wherein the peptide is an enzyme.

4. The method of claim 1 or 2 wherein the peptide is a short peptide consisting of less than 100 amino acid residues, preferably an antimicrobial peptide or an anti-tumor peptide.

5. The method of claim 4 wherein the peptide is an antimicrobial peptide or an antimicrobial enzyme active on bacteria, and the host cell is a bacterial cell (particularly *E. coli* or *Bacillus*), a filamentous fungus (particularly *Aspergillus*) or a yeast cell (particularly *Candida* or *Saccharomyces*).

6. The method of claim 4 wherein the peptide is an anti-tumor peptide, and the host cell is a mammalian cell, particularly a tumor cell.

7. The method of any of claims 4-6 wherein the peptide is a short peptide of less than 100 amino acid residues, and the ligation is such as to express the peptide without an extension or with an N-terminal extension of 1-5 amino acids and optionally a signal peptide.

8. The method of any of claims 1-7 wherein the selection of steps c) and e) is performed using agar plates

9. The method of any of claims 1-7 wherein the selection of steps c) and e) is performed in microtiter wells.

10. The method of any of claims 1-7 wherein the selection of steps c) and e) is performed using a FACS machine.

11. The method of any of claims 1-7 wherein the selection of steps c) and e) is performed by centrifugation or filtration.

12. Use of the method of any preceding claim for finding and preparing a composition for treatment of the human or animal body.

13. The method of claim 2, wherein the gene shuffling includes in vitro shuffling of homologous DNA comprising cleaving homologous template double-stranded polynucleotide into random fragments followed by homologously reassembling the fragments into full-length genes.

14. The method of claim 2, wherein the gene shuffling includes formation of a library of recombined homologous polynucleotides constructed from input DNA templates and random DNA primers by induced template shifts during in vitro DNA synthesis.

15. The method of claim 14, wherein the random DNA primers are employed to randomly initiate DNA synthesis on the mutant DNA templates that are to be combined.

16. The method of claim 2, wherein the gene shuffling includes using primers having less than 30 base pairs harboring the mutations to be combined.

17. The method of claim 2, wherein the gene shuffling includes synthesis of one or more degenerate DNA primers encoding an entire gene.

18. The method of claim 1, wherein the gene sequence diversity includes libraries of cDNA's or randomly generated whole-genome DNA fragments as starting material.

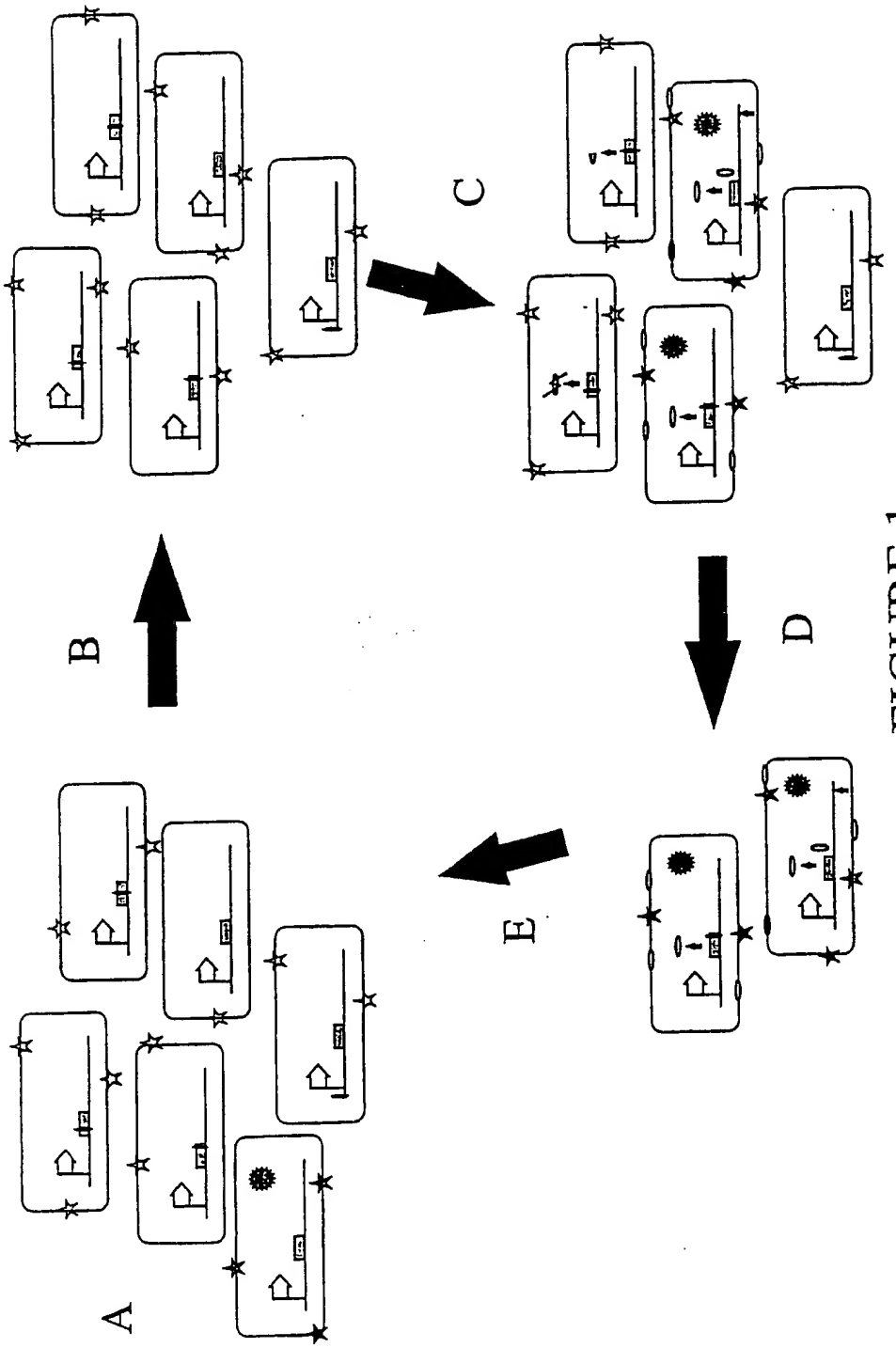


FIGURE 1

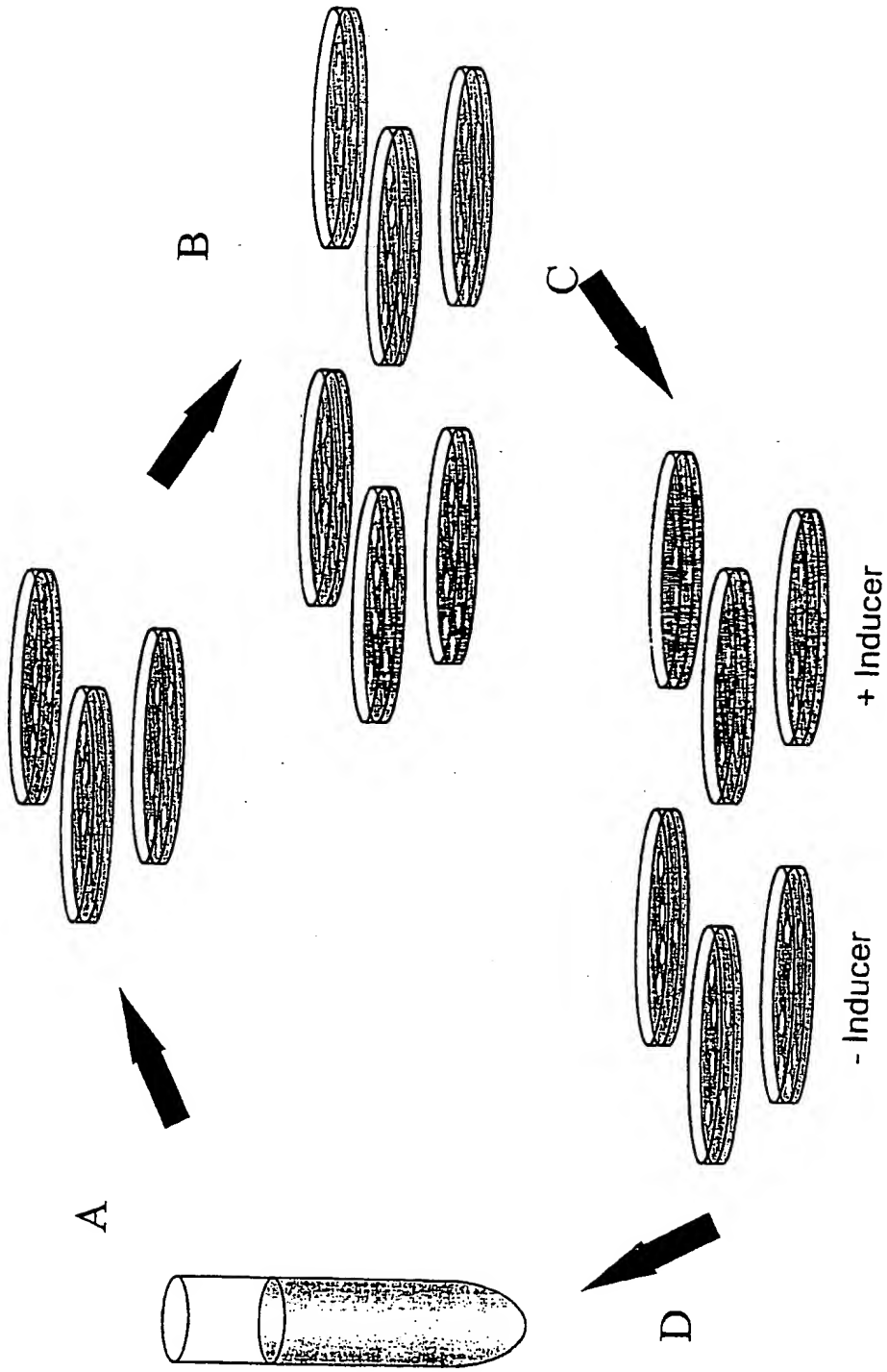
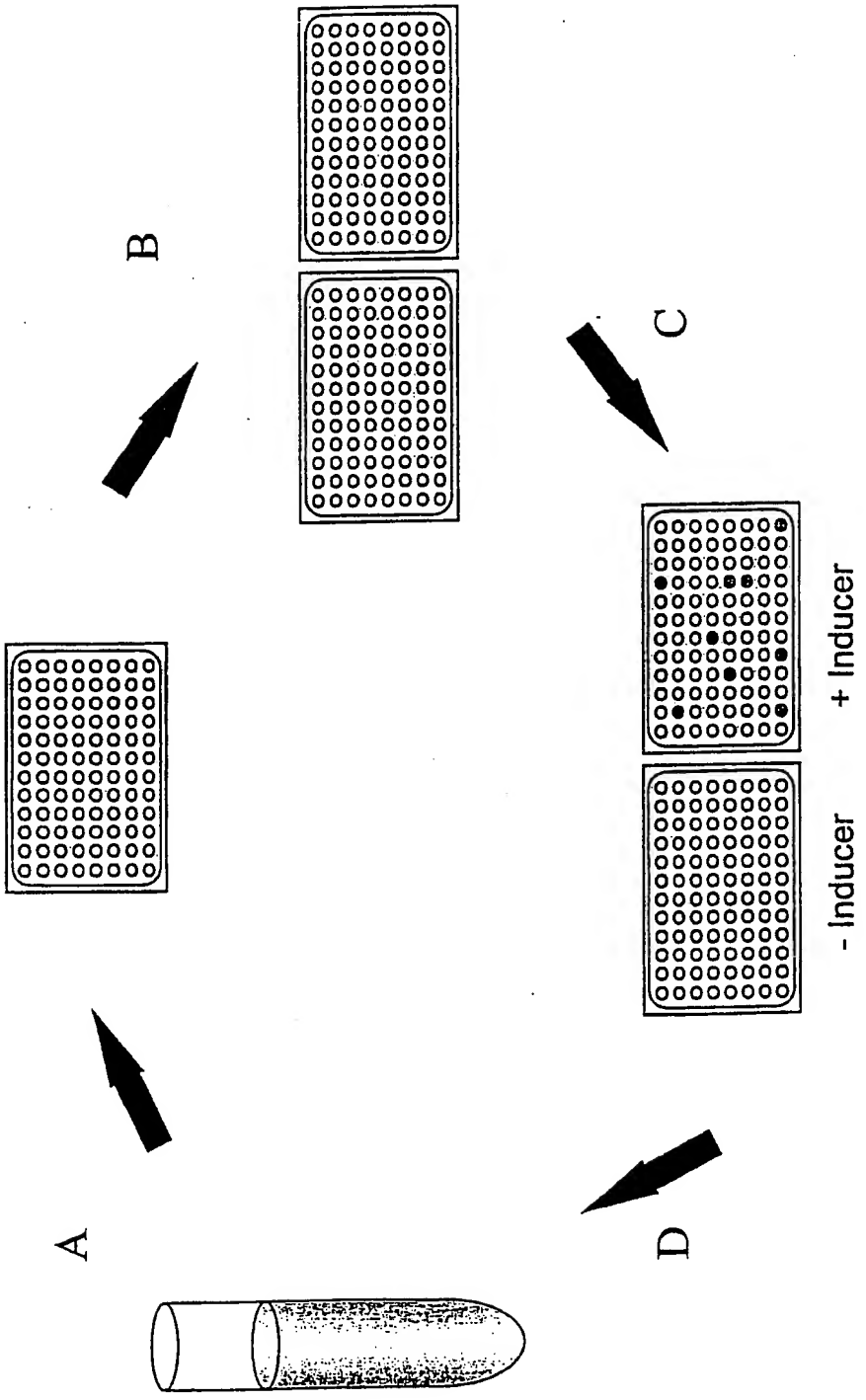


FIGURE 2



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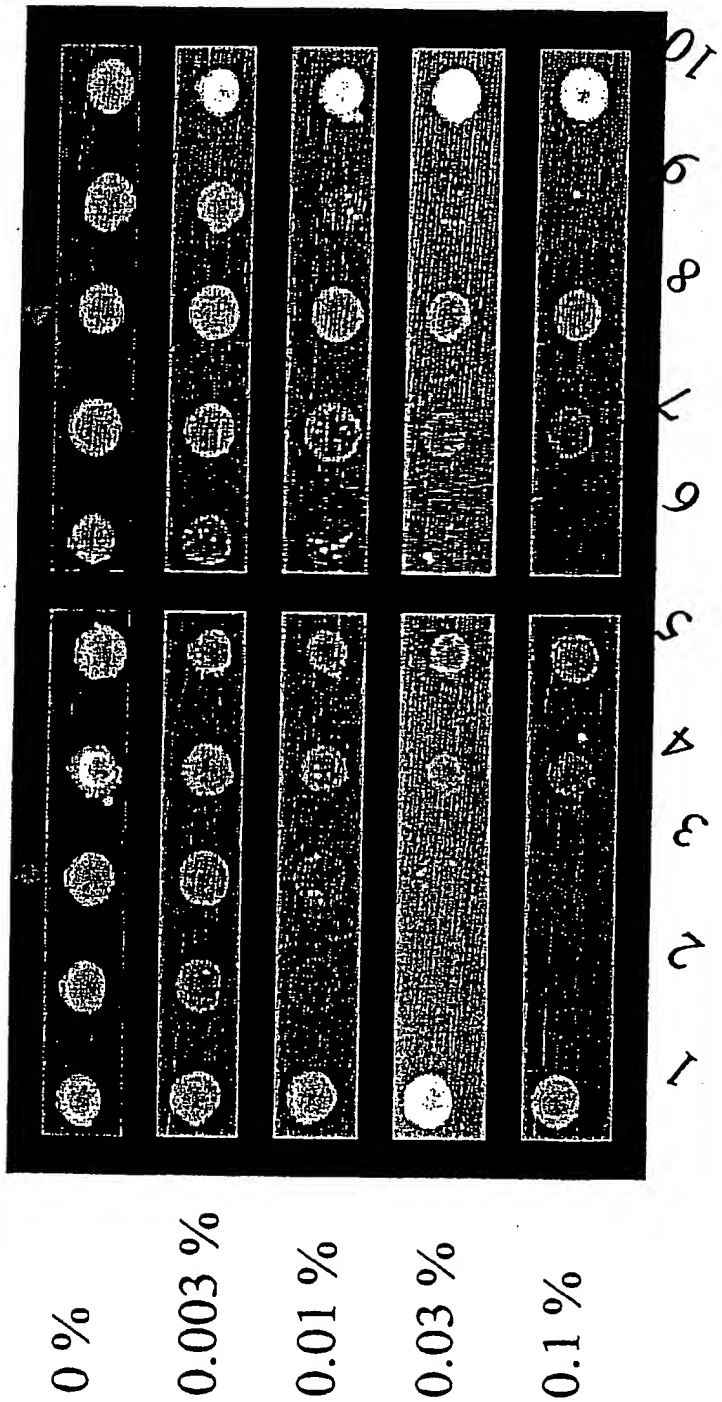


FIGURE 4

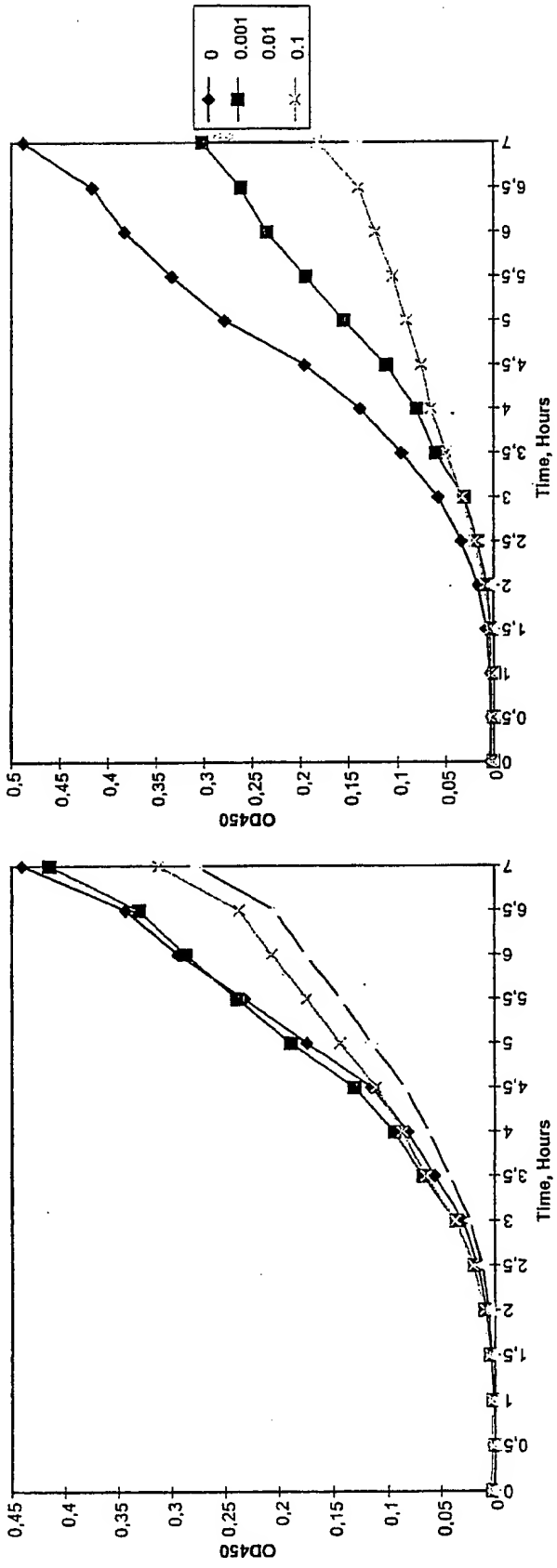


FIGURE 5

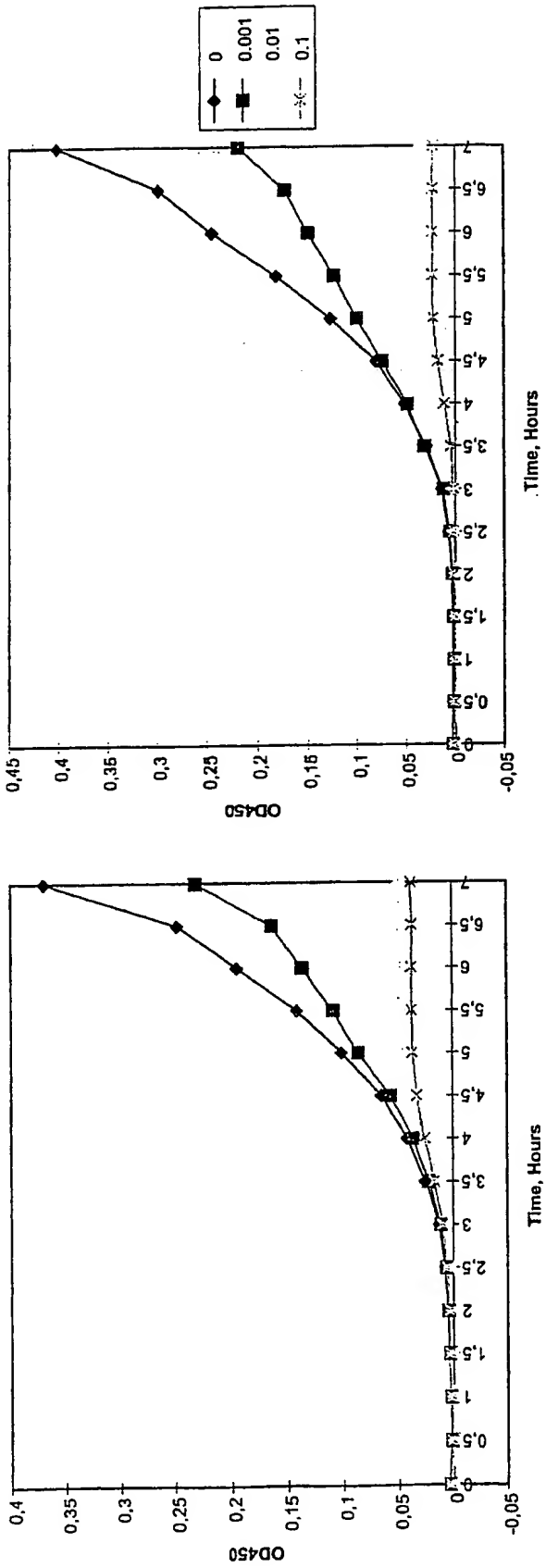


FIGURE 6

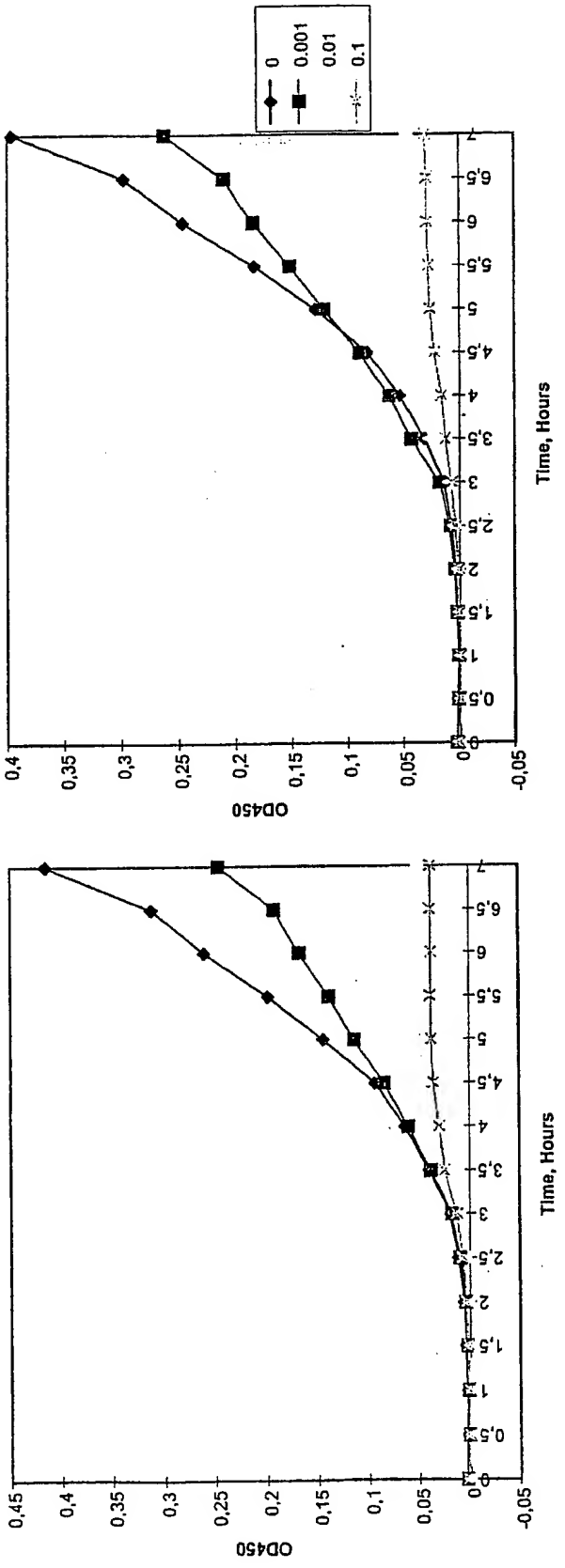


FIGURE 7

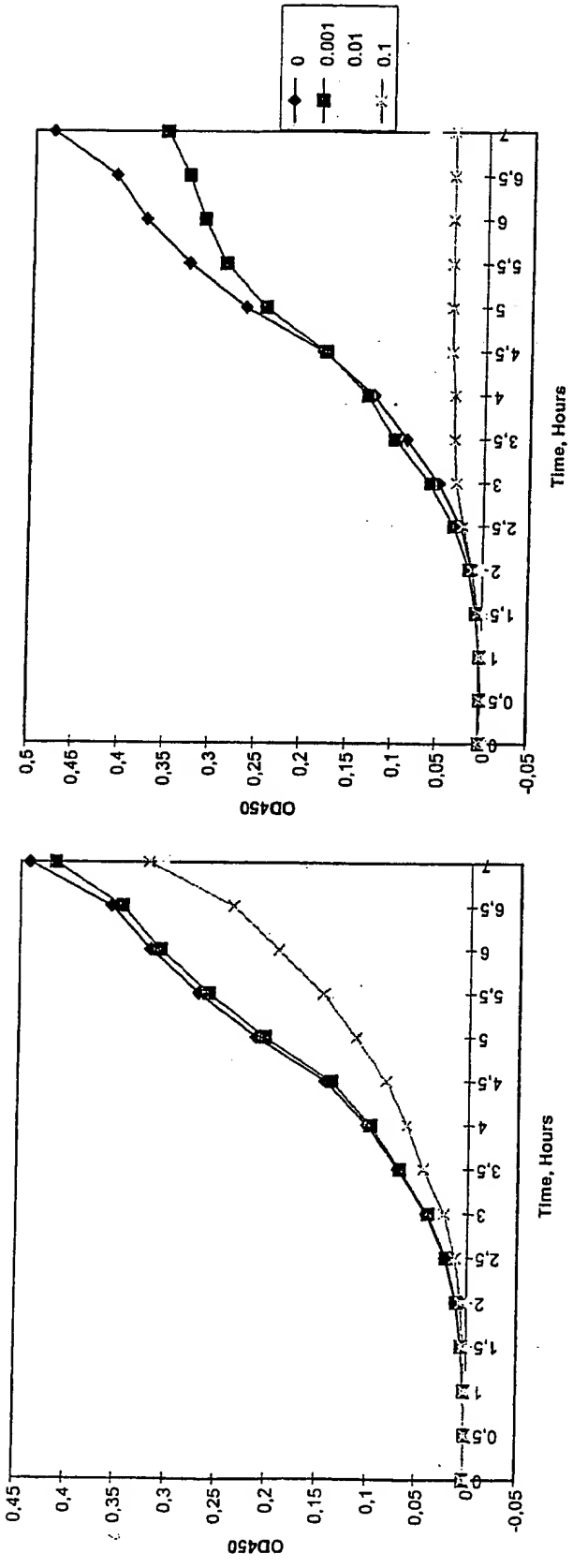


FIGURE 8

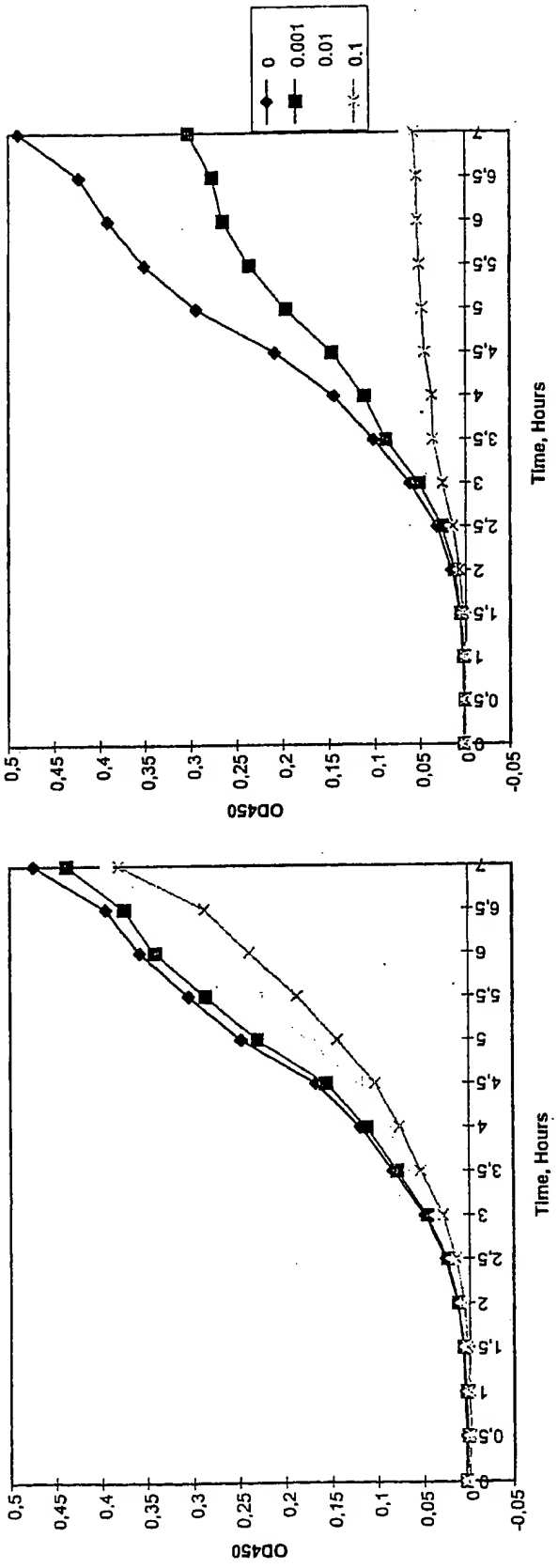


FIGURE 9

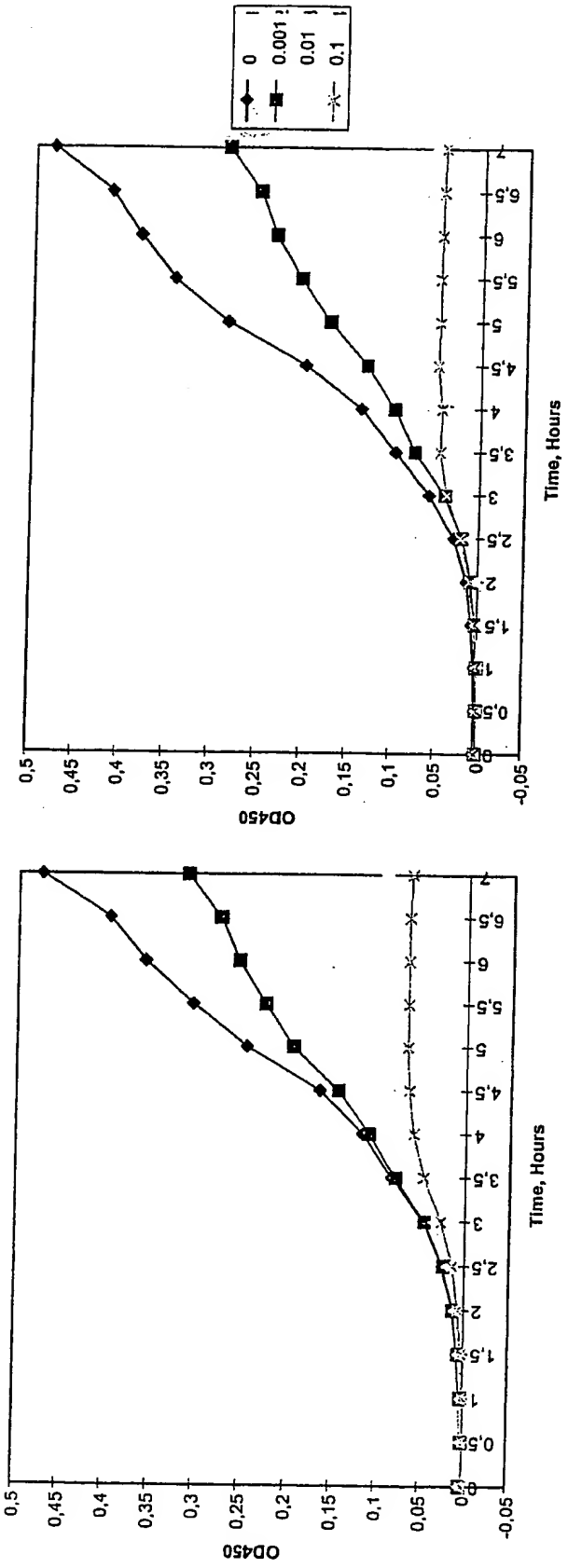


FIGURE 10

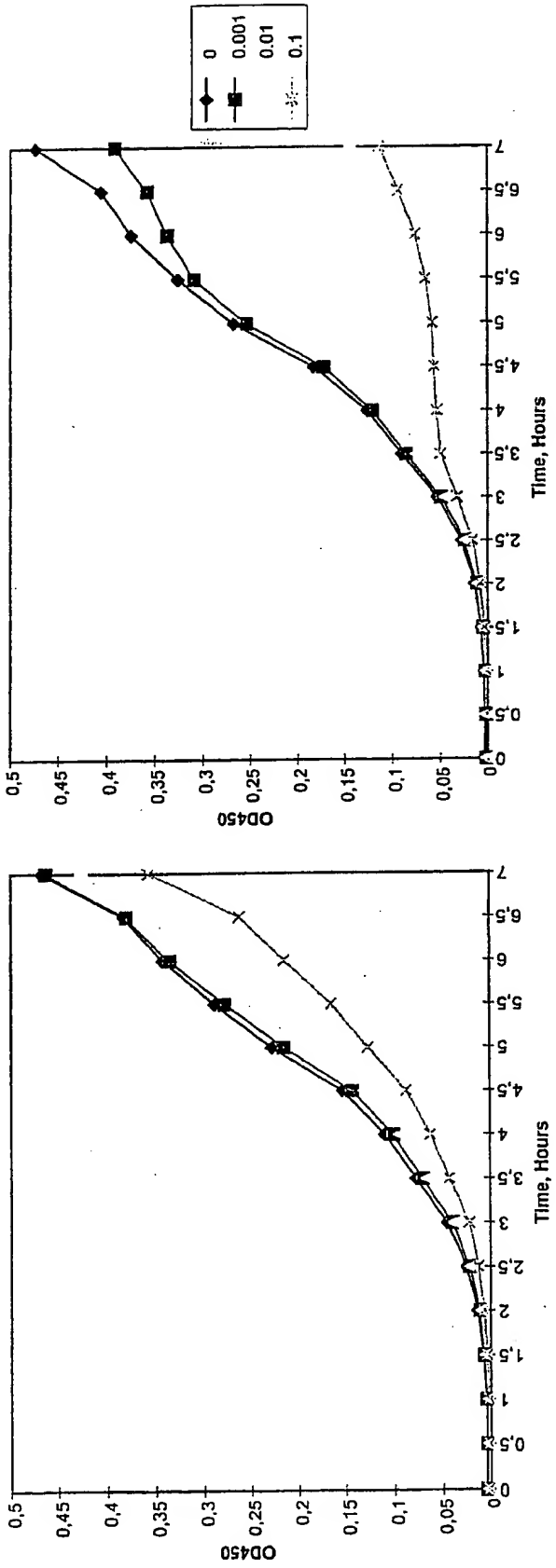
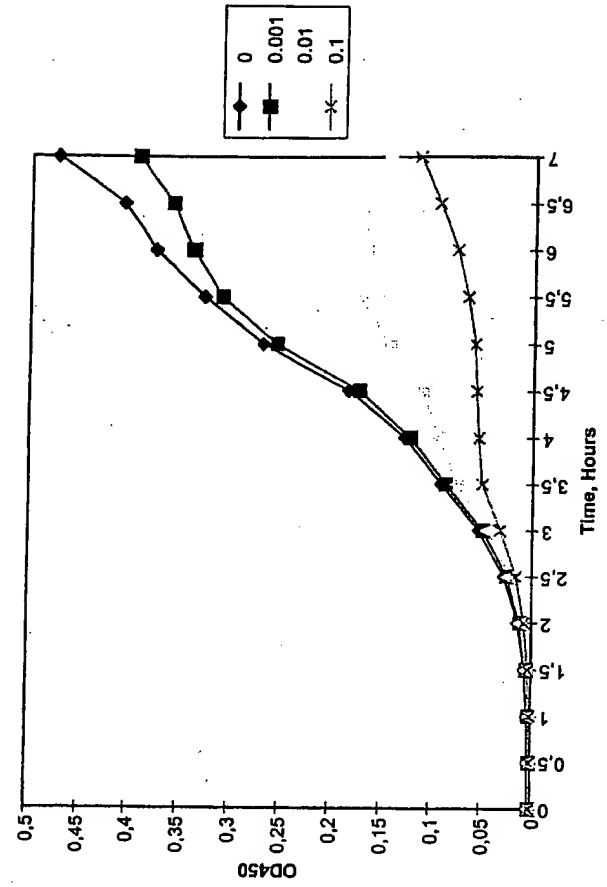
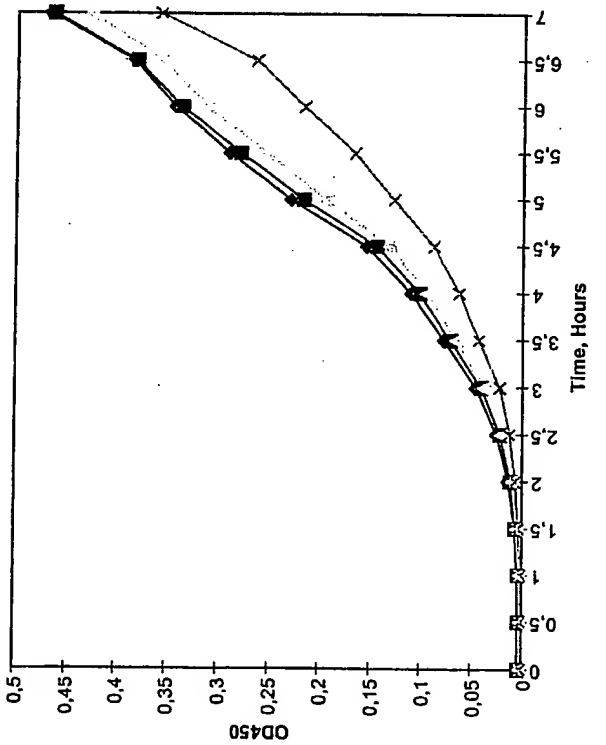


FIGURE 11



B



A

FIGURE 11

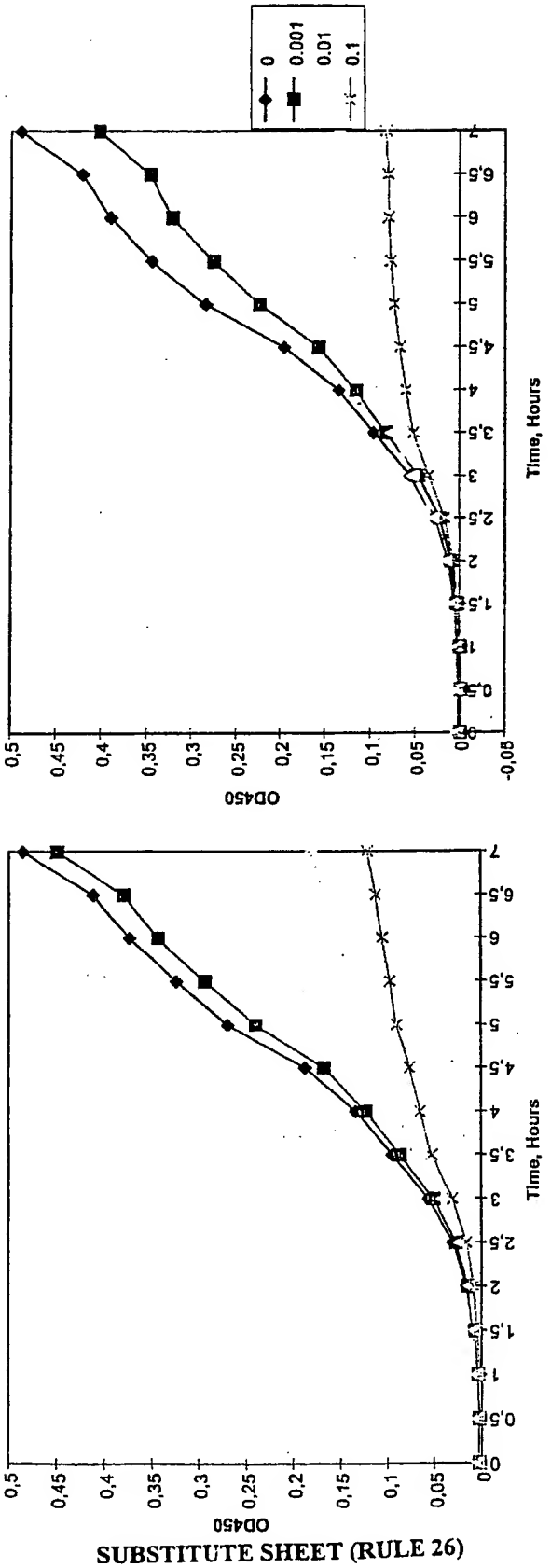


FIGURE 13

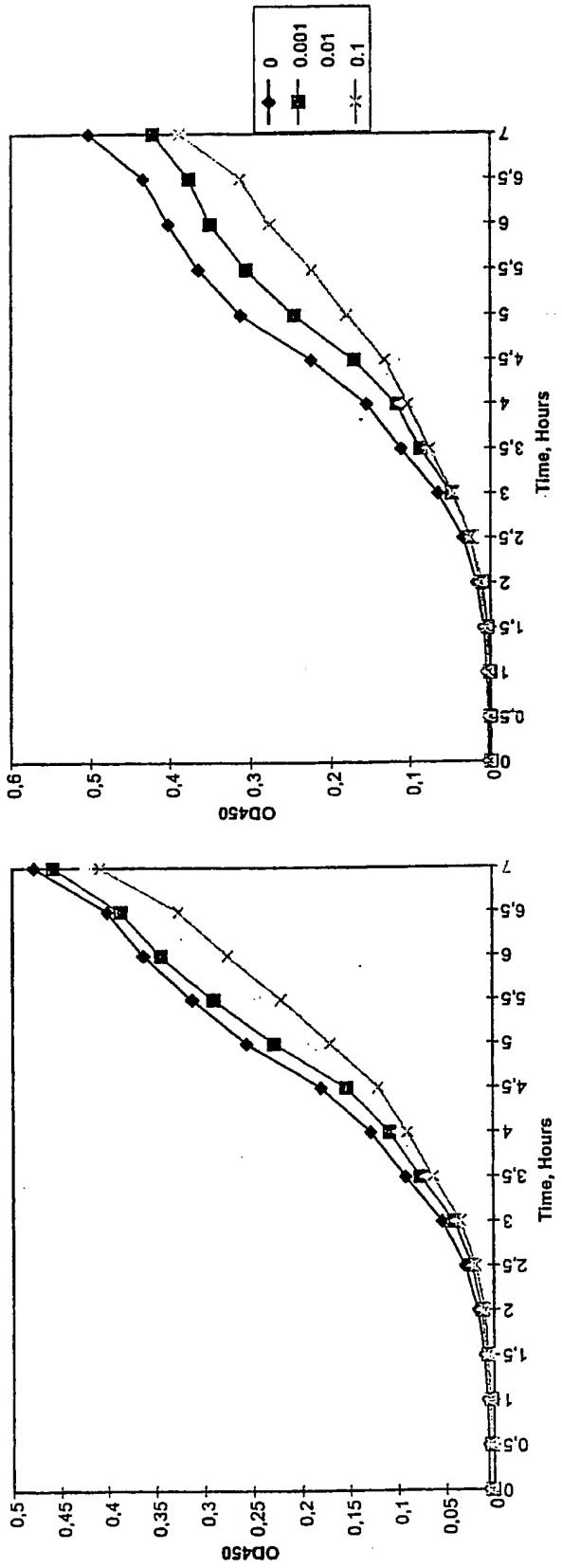


FIGURE 14

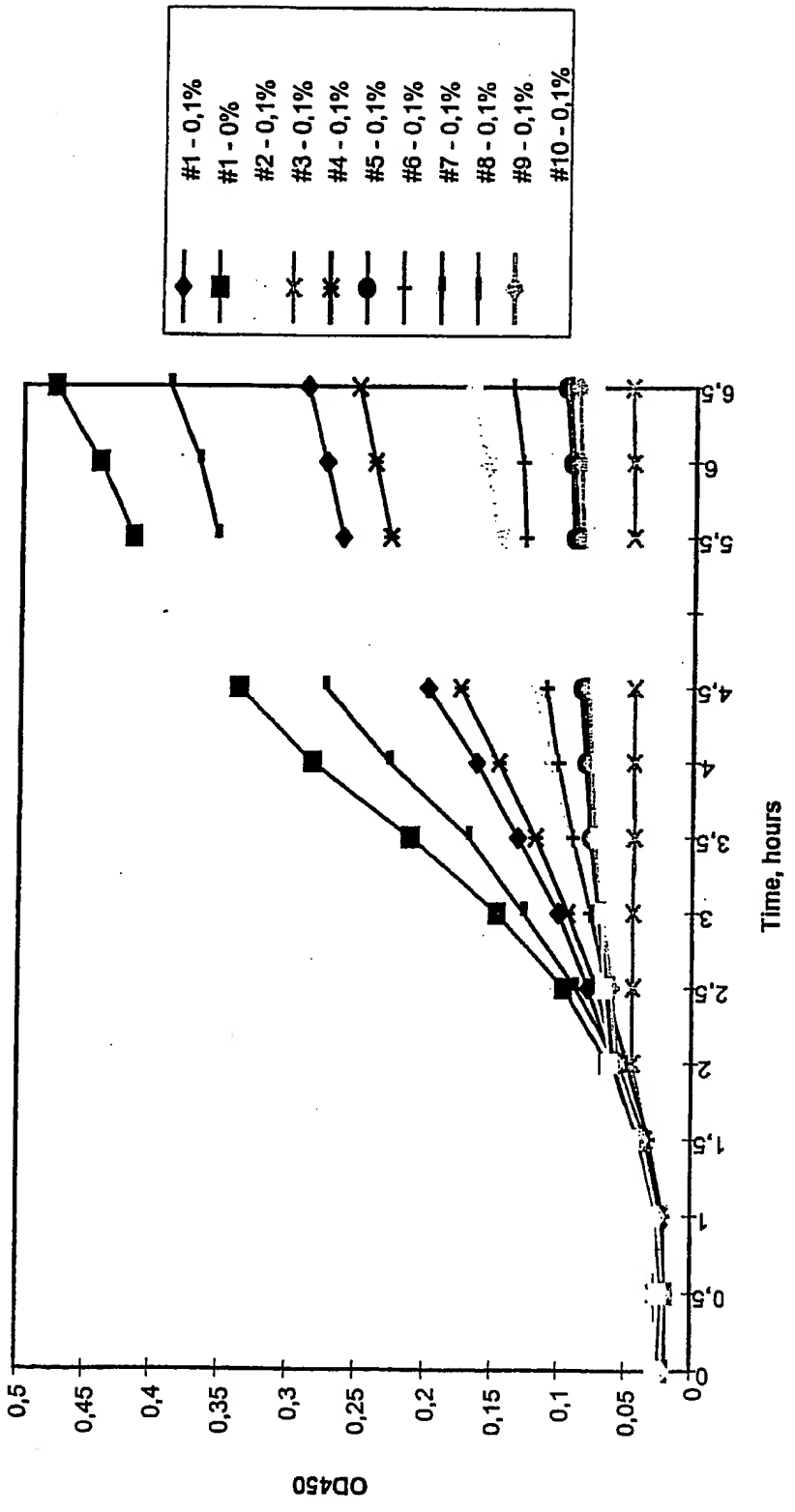

#1 - 0,1%
#1 - 0%
#2 - 0,1%
#3 - 0,1%
#4 - 0,1%
#5 - 0,1%
#6 - 0,1%
#7 - 0,1%
#8 - 0,1%
#9 - 0,1%
#10 - 0,1%
OD450
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
5,5
6
6,5
Time, hours

FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00287

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/10 // C12N 015/74, C12N 015/80, C07K 014/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9727213 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY), 31 July 1997 (31.07.97), page 21, line 7 - line 24; page 25, line 14 - line 24; page 31, line 27 - page 33, line 33, page 36, lines 5-10	1-12
Y		1-18
	--	
Y	Nature, Volume 370, August 1994, Willem PC Stemmer, "Rapid evolution of a protein in vitro by DNA shuffling", XP002082182	1-13,15-16
A		14,17-18
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 Sept 2000	18 -09- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson/gh Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00287

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9846796 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 22 October 1998 (22.10.98), see especially page 15, lines 3-6 and claims	1-12
Y		1-18
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Y	WO 9841653 A1 (NOVO NORDISK A/S), 24 Sept 1998 (24.09.98)	1-12,14-15
A		13,16-18
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Y	WO 9831837 A1 (MAXYGEN, INC.), 23 July 1998 (23.07.98)	1-12,17-18
A		13-16
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A		14,17-18
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INTERNATIONAL SEARCH REPORT

Information on patent family members

08/05/00

International application No.
PCT/DK 00/00287

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		EP 0877752 A	18/11/98
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